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Solid and liquid media for isolating and cultivating acidophilic and acid-tolerant sulfate-reducing bacteria

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Abstract

Growth media have been developed to facilitate the enrichment and isolation of acidophilic and acid-tolerant sulfate reducing bacteria (aSRB) from environmental and industrial samples, and to allow their cultivation *in vitro*. The main features of the "standard" solid and liquid devised media are: (i) use of glycerol rather than an aliphatic acid as electron donor; (ii) inclusion of stoichiometric concentrations of zinc ions to both buffer pH and to convert potentially harmful hydrogen sulfide produced by the aSRB to insoluble zinc sulfide; (ii) inclusion of *Acidocella aromatica* (an heterotrophic acidophile that does not metabolize glycerol or yeast extract) in the gel underlayer of double layered (overlay) solid media, to remove acetic acid produced by aSRB that incompletely oxidize glycerol and also aliphatic acids (mostly pyruvic) released by acid hydrolysis of the gelling agent used (agarose). Colonies of aSRB are readily distinguished from those of other anaerobes due to their deposition and accumulation of metal sulfide precipitates. Data presented illustrate the effectiveness of the overlay solid media described for isolating aSRB from acidic anaerobic sediments and low pH sulfidogenic bioreactors.

Introduction

Sulfate reducing bacteria (SRB) comprise a large number of phylogenetically-diverse prokaryotes that have in common their ability to grow by catalyzing the dissimilatory reduction of sulfate to sulfide in anoxic environments (Muyzer & Stams, 2008). They have been detected in, and isolated from, many different marine and freshwater environments, and soils, and also from many "extreme" environments. Thermophilic, psychrophilic, halophilic and alkalophilic species have been described. Acidic environments have presented something of a conundrum, however, as although there have been numerous reports of sulfidogenic activity in anoxic sediments in, for example, sulfate-rich streams draining mine

sites, attempts to isolate acidophilic or acid-tolerant strains of SRB (aSRB) have, until relatively recently, mostly been unsuccessful (Dopson & Johnson, 2012).

One of the hazards faced by SRB is that the sulfide they produce (particularly H_2S, which is the dominant form present at pH < 7) is toxic to them, as to other life forms, at relatively low (milli-molar) concentrations (Koschorreck, 2008). In many situations (e.g. enrichment culture formulations for neutrophilic SRB) ferrous iron acts as a sink for sulfide, removing it as insoluble, non-bioavailable, iron sulfide (FeS). However, the solubility product of FeS is such that it does not precipitate at pH <5, and therefore cannot act as an effective sink for sulfide in moderately to extremely acidic situations (Lewis, 2010). A second generic problem for acidophiles is the toxicity of small molecular weight organic acids (Ingledew & Norris, 1992). Many SRB are “incomplete substrate oxidizers”, in that they partially oxidize organic substrates and release the end product(s) of their metabolism (frequently acetate) into their growth milieu. At pH values below their respective pK_a values (4.75 for acetic acid) small molecular weight organic acids occur predominantly as non-dissociated, lipophilic molecules. Acetic acid is toxic to many chemolithotrophic bacteria when present in micro-molar concentrations, though some heterotrophic acidophiles (e.g. *Acidocella* (*Ac.*) *aromatica*) are more tolerant of this and some other aliphatic acids, and use it as a carbon and energy source (Jones *et al*., 2013). Many media formulations used to enrich neutrophilic SRB utilize organic acids, such as lactate, as carbon and energy sources (e.g. Postgate, 1963). As with acetic acid, lactate exists predominantly as non-dissociated lactic acid at low pH, and again micro-molar concentrations of this potential electron donor are sufficient to partially or completely inhibit the growth of SRB (and most other bacteria) in acidic media. In contrast, organic substrates, such as glycerol, that are uncharged at low pH have been used successfully to enrich for acid-tolerant strains (e.g. Sen & Johnson, 1999).

A variety of solid media formulations have been developed to facilitate the isolation and enumeration of acidophilic chemolithotrophic and heterotrophic microorganisms from environmental and industrial samples (Johnson & Hallberg, 2007). Most of these media employ an “overlay” technique, in which a double-layered gel is used in a standard Petri

plate, the lower layer of which is inoculated with an active culture of a heterotrophic acidophile (usually an *Acidiphilium* (*A.*) sp.) while the upper layer is not. The rationale is that the heterotrophic acidophile metabolizes the small molecular weight compounds (such as pyruvic acid) that derive from acid hydrolysis of commonly-used gelling agents such as agar. Using this technique, it has been possible to routinely isolate and cultivate chemolithotrophic bacteria (such as *Leptospirillum* spp.) that had previously been considered not to grow on solid media (Johnson, 1995).

Here we describe solid and liquid media that have been developed in the authors' laboratory and used successfully over a number of years to isolate and enumerate aSRB from environmental samples, and also to cultivate isolates as axenic cultures in the laboratory.

Materials and methods

Solid media

The standard solid medium developed to isolate aSRB from environmental samples and laboratory enrichment cultures was a variant of overlay media previously described for aerobic acidophiles (Johnson & Hallberg, 2007). The main differences were (i) higher pH of the "standard" aSRB medium (~3.7 compared to ~ 2.7 for the aerobic medium plates); (ii) using the type strain of *Ac. aromatica* rather than *A. cryptum* strain SJH in the underlay gel; (iii) inclusion of 4 mM glycerol (as electron donor) and 7 mM zinc (as the sink for hydrogen sulfide). Two solutions were prepared and sterilised separately by autoclaving (121˚C, 20 mins) and a third solution (acidic ferrous sulfate) sterilized by filtration through 0.2 µm (pore size) membranes:

Solution A - 20 mL of concentrated basal salts solution, containing (g/L) $MgSO_4 \cdot 7H_2O$ (25), $(NH_4)_2SO_4$ (22.5), $Na_2SO_4 \cdot 10H_2O$ (7.5); KH_2PO_4 (2.5), KCl (2.5) and $Ca(NO_3)_2 \cdot 4H_2O$

(0.7), mixed with 770 mL of reverse osmosis (RO)-grade water, 4 mL of a 1 M glycerol solution, 0.1 g of yeast extract, 7 mL of 1 M zinc sulfate and 0.875 g of magnesium sulfate. One millilitre of a concentrated trace elements solution was added, the mixture adjusted to pH 3.5 with sulfuric acid, and autoclaved. The trace elements solution contained (g/L): $ZnSO_4 \cdot 7H_20$ (10); $CuSO_4 \cdot 5H_2O$ (1.0); $MnSO_4 \cdot 4H_2O$ (1.0); $CoSO_4 \cdot 7H_2O$ (1.0); $Cr_2(SO_4)_3 \cdot 15H_2O$ (0.5); H_3BO_3 (0.6); $Na_2MoO_4 \cdot 2H_2O$ (0.5); $NiSO_4 \cdot 6H_2O$ (1); $Na_2SeO_4 \cdot 10H_2O$ (1); $Na_2WO_4 \cdot 2H_2O$ (0.1); $NaVO_3$ (0.1).

Solution B - 5 g of agarose (Sigma Type I) suspended in 200 mL of RO water.

Solutions A and B were combined when they had cooled to about 50°C, and 0.1 mL of 1 M ferrous sulfate (*Solution C*) added. The combined solutions were mixed and split into two portions of approximately similar volumes. Ten millilitres of a culture of *Ac. aromatica*^{T} (pre-grown in an acidic (pH 3) medium containing 5 mM fructose, basal salts and trace elements (as above) was added to one portion, and *ca*. 20 mL aliquots of the inoculated molten gel poured into sterile Petri plates. When this layer had solidified, the other (sterile) combined solution, which had been retained in a molten state by storing in a 50˚C water bath, was poured on top, again in aliquots of *ca*. 20 mL/plate (Fig. 1).

Variants on this “standard” (glycerol/zinc) aSRB solid medium formulation were: (i) a non-overlay version; (ii) one in which zinc sulfate was replaced with 7 mM (final concentration) ferrous sulfate; (iii) more acidic variants (final pH values of ~2.8 or 2.3, achieved by adjusting the pH of solution A to either 2.5 or 2.0, respectively) in which zinc sulfate was replaced with copper sulfate (CuS has a smaller solubility product than ZnS and precipitates at pH 2.8 whereas ZnS does not). Because of the greater sensitivity of *Ac. aromatica* to copper than to zinc (Jones *et al*., 2013) the concentration of copper sulfate added was 0.25 mM; (iv) a circum-neutral pH non-overlay variant (adjusting the pH of solution A to 7.0). The pH values of the gelled media were measured using a calibrated flat-tipped combined pH electrode (Hanna instruments, UK) coupled to an Accumet 50 pH/E_H meter.

Liquid media

The standard liquid medium used to grow aSRB isolates *in vitro* contained basal salts/trace elements/magnesium sulfate (as above) supplemented with 4 mM (final concentration) glycerol, 0.01% (w/v) yeast extract and 7 mM zinc sulfate. The pH of the medium was adjusted to either 3.5 or 4.0 with 1 M sulfuric acid, de-aerated under vacuum, and autoclaved at 121˚C for 20 minutes. Ferrous sulfate was added to the cooled medium to give a final concentration of 100 µM (resulting in slight reductions in media pH, to ~ 3.4 and 3.7, respectively). The liquid medium was stored at ambient temperature in an anaerobic glove-box (Plas Labs, Lansing, MI) under a nitrogen/carbon dioxide atmosphere prior to use. A second liquid medium in which zinc sulfate was replaced with 7 mM ferrous sulfate (and pH adjusted between 3.0 and 5.0) was also used in some experiments.

Isolation of aSRB on solid media

(i) Environmental samples. Sediments in an acidic stream draining an abandoned copper mine (Cantareras, located in the Iberian Pyrite Belt in south-west Spain; Rowe *et al*., 2007) were sampled at the entrance, and 10, 30 and 60 m downstream of the mine adit. Sediment samples were placed in sterile Falcon tubes, which were filled to capacity and sealed at the mine site. In the laboratory, 0.5 g of each sediment was mixed with 0.5 ml of pH 2.5 basal salts solution and the suspensions serially diluted and spread onto overlay aSRB solid media. Inoculated plates were incubated under anaerobic conditions at 30°C using the AnaeroGen system (Oxoid, U.K.), for up to one month. Colonies that grew on SRB plates were examined under a binocular microscope, and sulfidogens tentatively identified by their deposition of metal sulfides (ZnS or CuS) which gave distinctive coloration and metallic sheens to the colonies (Fig. 2). The identities of putative aSRB isolates were confirmed by amplifying and sequencing their 16S rRNA genes (Rowe *et al*., 2007). Other overlay plates, formulated to support the growth of chemolithotrophic and heterotrophic acidophiles

(Johnson & Hallberg, 2007) were inoculated at the same time and incubated at 30˚C under aerobic conditions.

(ii) Laboratory bioreactor cultures. Liquid samples from two continuous-flow sulfidogenic bioreactors, maintained at between pH 2.8 and 4.5 and used to selectively precipitate transition metals from synthetic mine waters (Ňancucheo & Johnson, 2012a) were serially-diluted and spread onto glycerol/zinc overlay aSRB medium. Terminal restriction enzyme fragment length polymorphism (T-RFLP) analysis was carried out in parallel to obtain an overview of the compositions of the bacterial communities in the bioreactors (Ňancucheo & Johnson, 2012a). The bioreactors had originally been inoculated with pure cultures of two aSRB: *Desulfosporosinus acididurans*^{T} (Sánchez-Andrea *et al*., 2015) and "*Desulfobacillus acidavidus*" strain CL4 (Ňancucheo & Johnson, 2012a), and also with dissected samples of a sulfidogenic microbial mat taken from a stream draining the Cantareras mine.

(iii) Pure cultures of aSRB. Acidophilic and acid-tolerant SRB that had been isolated previously were also tested for growth on the various solid media. These were *D. acididurans*^{T} (originally isolated from a geothermal site in Monserrat, West Indies), and "*Db. acidavidus*" strain CL4 and *Firmicute* strain C5, both of which had been isolated from the microbial mat at Cantareras.

Results and discussion

The solid and liquid media described have been used routinely to isolate and cultivate aSRB in the authors' laboratory for several years. *Ac. aromatica* is used in the underlayer gel, as, unlike *Acidiphiliium* spp and other *Acidocella* spp., this heterotrophic acidophile uses only a limited range of organic donors (including fructose and acetic acid) but does not grow on yeast extract, glucose, glycerol or many other small molecular weight organic compounds that are commonly metabolized by acidophilic heterotrophic microorganisms. The aSRB present in plate inocula and *Ac. aromatica* do not compete, therefore, for the glycerol and

yeast extract included in the aSRB overlay media. However, acetic acid, which is produced as a metabolic waste product by some strains of aSRB, is metabolized by the non-sulfidogenic heterotrophic acidophile, thereby circumventing the problem of this (and other) aliphatic acids inhibiting the growth of the sulfate-reducers. Both the standard solid and liquid media contain zinc sulfate, which has two important functions: (i) removal of toxic hydrogen sulfide as ZnS and (ii) buffering pH. In acidic media, sulfidogenesis is a proton-consuming reaction, as (a) the end products of organic substrate (e.g. glycerol) oxidation are either carbon dioxide (complete oxidizers; equation [1]) or a mixture of carbon dioxide and acetic acid (incomplete oxidizers; equation [2]), and (b) sulfate is reduced to H_2S rather than to HS^-:

$$4\ C_3H_8O_3 + 7\ SO_4^{2-} + 14\ H^+ \rightarrow 12\ CO_2 + 7\ H_2S + 16\ H_2O \qquad [1]$$

$$4\ C_3H_8O_3 + 3\ SO_4^{2-} + 6\ H^+ \rightarrow 4\ CH_3COOH + 4\ CO_2 + 3\ H_2S + 8\ H_2O \qquad [2].$$

However, the reaction between soluble zinc ions and hydrogen sulfide generates protons (equation [3]), which causes the net reaction in the presence of zinc (shown for the complete oxidation of glycerol in equation [4]) to be pH-neutral:

$$Zn^{2+} + H_2S \rightarrow ZnS + 2\ H^+ \qquad [3]$$

$$4\ C_3H_8O_3 + 7\ SO_4^{2-} + 7\ Zn^{2+} \rightarrow 12\ CO_2 + 7\ ZnS + 16\ H_2O \qquad [4].$$

For this to be effective, soluble zinc has to be present in greater or equimolar concentrations to the amount of hydrogen sulfide produced. The “standard” solid and liquid media devised for isolating aSRB contain a molar ratio of glycerol and zinc of 4:7, which provides sufficient zinc to react with the theoretical maximum amount of H_2S generated (i.e. for aSRB that oxidize glycerol completely to CO_2) and an excess in the case of incomplete oxidizers. All strains of aSRB isolated in the authors’ laboratory have been found to tolerate zinc concentrations well above 7 mM, so that the issue of potential zinc toxicity does not arise. However, lower concentrations of zinc can also be used (if toxicity is suspected) in both solid

and liquid media, so long as the 4:7 ratio (assuming that glycerol is used as electron donor) is maintained. Measurement of the pH of solid and liquid glycerol/zinc media confirmed that pH changes were minor (increasing from ~ 3.7 to ~ 4.0) in grown cultures, in contrast to those containing ferrous sulfate where the pH increased to ~7. The presence of zinc in both solid and liquid media also served as a useful indicator of growth of aSRB. Formation of ZnS in liquid cultures is evidenced by the formation of silver/pink-coloured metallic precipitates (which tend to coat the walls of growth vessels), while ZnS-stained colonies of aSRB are also readily differentiated from non-sulfidogenic anaerobes. Continued accumulation of ZnS causes aSRB colonies to develop hard surface coats with protracted incubation (Fig. 2e).

Isolation of aSRB from environmental samples.

Figure 3 shows data of direct isolation of bacteria from stream sediments taken from the abandoned Cantareras copper mine using a variety of overlay solid media. Isolates were categorized as: (i) iron-oxidizing aerobes (ferric iron-encrusted colonies on aerobically-incubated ferrous iron overlay plates); (ii) heterotrophic aerobes (colonies that grew on aerobically-incubated yeast extract overlay plates): (iii) aSRB (colonies encrusted with ZnS or CuS on anaerobically-incubated overlay plates); (iv) other anaerobes (colonies on anaerobically-incubated overlay plates that were not encrusted with ZnS or CuS). Confirmation of sulfide production by putative SRB isolates was confirmed when colonies were transferred into liquid media, and isolates identified from sequence analysis of their 16S rRNA genes.

Numbers of cultivatable acidophiles were relatively low in the sediment sample from the mine adit entrance ($<10^3$/g) but were generally far greater (in some cases by 2-3 orders of magnitude) downstream of the adit. This was particularly noticeable with the anaerobic acidophiles (both the aSRB and the non-sulfidogenic isolates) and corresponded to samples underlying streamer/mat growths that proliferated in the drainage channel at those sampling points (Rowe *et al*., 2007). The surfaces of these stratified macroscopic growths were

dominated by acidophilic algae which were thought to provide much of the organic carbon that sustained the underlying streamer/mat microbial communities, which were predominantly heterotrophic (Ňancucheo & Johnson, 2012b). The identities of the non-sulfidogenic anaerobic acidophiles were not determined.

Colony forming units of putative aSRB were about an order of magnitude fewer on glycerol/copper plates (pH 2.8) than on the higher pH zinc-containing plates (sediments taken at 10 and 30 m downstream of the adit entrance) but were present in slightly greater numbers in the other two sediment samples (Fig. 3). No colonies grew on the more acidic (pH 2.3) glycerol/copper plates nor on the circum-neutral pH solid medium. Colonies of aSRB on copper-containing plates (stained black due to the deposition of CuS) were much smaller than those on zinc-containing plates and displayed superior growth when transferred to the latter. This was considered due, at least in part, to the greater amount of electron donor (glycerol) in the “standard” solid medium, but could also reflect a preference of the isolates for growing at slightly higher pH.

Isolation of aSRB from laboratory bioreactor samples

Bacteria (both aSRB and non-sulfidogens) were isolated on solid media at regular intervals from acidic sulfidogenic bioreactors used to selectively remove transition metals from synthetic acidic mine drainage waters and operated at different pH values (Ňancucheo & Johnson, 2012a; Hedrich & Johnson, 2014). Figure 4 shows T-RFLP profiles of the bacterial populations when one of the bioreactors was operated at pH 4.5, 3.0 and 2.8. The profiles were dominated by four restriction fragments of different lengths, each corresponding to a single bacterial species. Two of these were acidophilic sulfidogens (*D. acididurans* and *Peptococcaceae* strain CEB3; Petzsch *et al*., 2015) while the other two (*Acidithiobacillus ferrooxidans* and *Alicyclobacillaceae* strain IR2) were facultative anaerobes that did not reduce sulfate. The two sulfidogens and strain IR2 were all isolated from bioreactor liquors on glycerol/zinc plates incubated under anaerobic conditions, while *At. ferrooxidans* was

isolated on ferrous iron-containing overlay plates incubated aerobically (Johnson & Hallberg, 2007). Plate counts reflected the relative abundance of bacteria indicated by semi-quantitative T-RFLP analysis.

Growth of pure cultures of aSRB on solid media

Some strains of aSRB isolated and maintained in the authors' laboratory (e.g. strains CL4 and C5) grew on both overlay and non-overlay glycerol/zinc solid media, though colonies tended to grow more slowly and were smaller on the latter. In contrast, *D. acididurans* only grew on the overlay variant of the standard medium, though it also grew on non-overlay plates in which ferrous iron substituted for zinc sulfate. In the latter case, the colonies of *D. acididurans* were heavily blacked-stained (encrusted with FeS) rather than encrusted with silver/pink-coloured ZnS (Figs. 2a & 2b) and the pH of the solid medium increased to ~7 rather than being buffered at ~ pH 4. These differences in behaviour can be attributed to the fact that strains CL4 and C5 appear to oxidize glycerol completely to CO_2, whereas *D. acididurans* is an incomplete oxidizer. In the pH-buffered glycerol/zinc plates, the acetic acid produced by *D. acididurans* inhibits the growth of the sulfidogen, unless (as in the overlay plate variant) it is removed by *Ac. aromatica* (Kimura *et al*., 2006). However, in the absence of zinc, the pH of the medium increased (as evidenced by the formation of FeS) causing acetic acid to dissociate to the relatively non-toxic acetate anion, thereby allowing this sulfidogen to grow. Overlay plates are considered to be more versatile and efficient, particularly for isolating acidophilic sulfidogens from environmental samples, given that these may contain both complete and incomplete substrate-oxidizing aSRB.

While the solid and liquid media described herein have proven effective for isolating and cultivating aSRB, other formulations have also been used with varying success. Many sulfidogens (including *D. acididurans*), for example, can use hydrogen as electron donor, and in this case the potential problem of toxicity caused by incomplete oxidation of an organic electron donor obviously does not arise. There are several pragmatic issues, however, which

can limit the use of this inorganic electron donor, including the fact that, unlike with glycerol, it is extremely difficult or impossible to control the molar ratio of this electron donor to the H_2S sink (zinc ions) which severely limits the potential for pH buffering in batch liquid cultures and solid media. Experiments carried out in which glycerol/zinc plates incubated in anaerobic jars with or without hydrogen gas has not resulted in any significant increases in numbers of aSRB colonies obtained from environmental samples, suggesting that many, if not most hydrogen-oxidizing aSRB, can also grow on glycerol.

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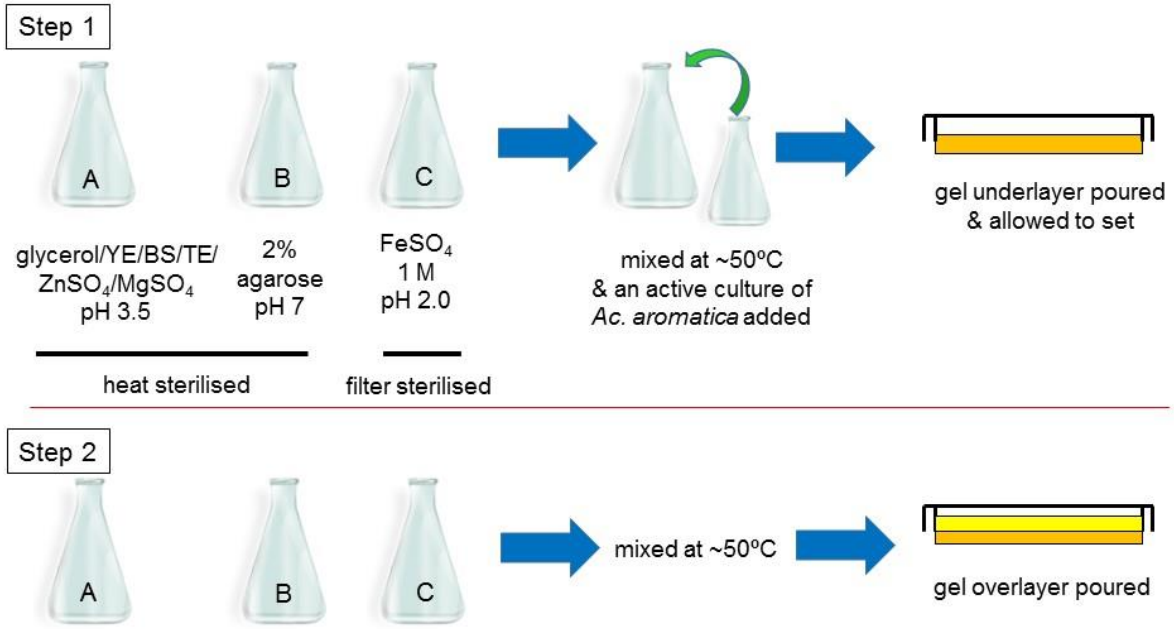

Fig. 1. Schematic representation of the approach used to prepare overlay medium for aSRB

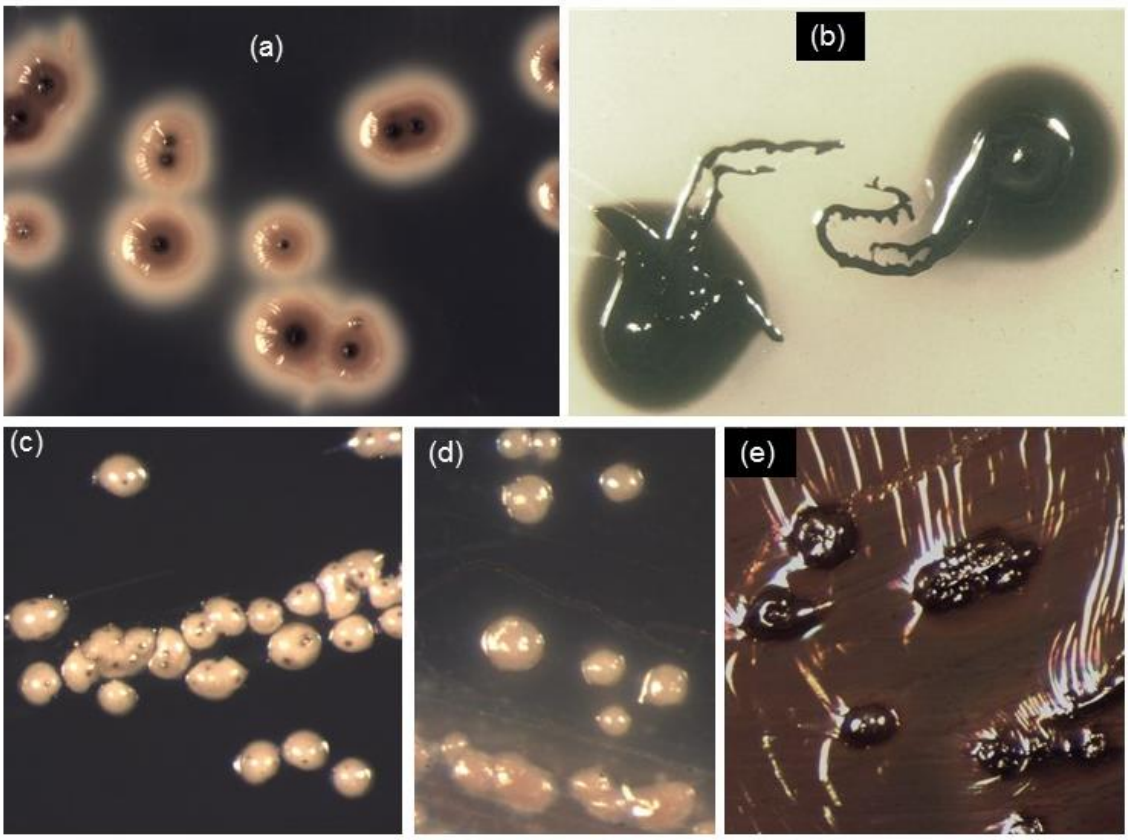

Fig. 2. Colonies of aSRB grown on solid media: (a) *D. acididurans*, grown on glycerol/zinc overlay medium; (b) *D. acididurans* grown on glycerol/ferrous iron non-overlay medium; (c) "*Db. acidavidus*" strain CL4, grown on glycerol/zinc overlay medium; (d) *Peptococcaceae* strain CEB3, grown on glycerol/zinc overlay medium; (e) *Firmicute* strain C5 grown on glycerol/zinc overlay medium for 6 weeks, showing colonies covered with shell-like coating of ZnS.

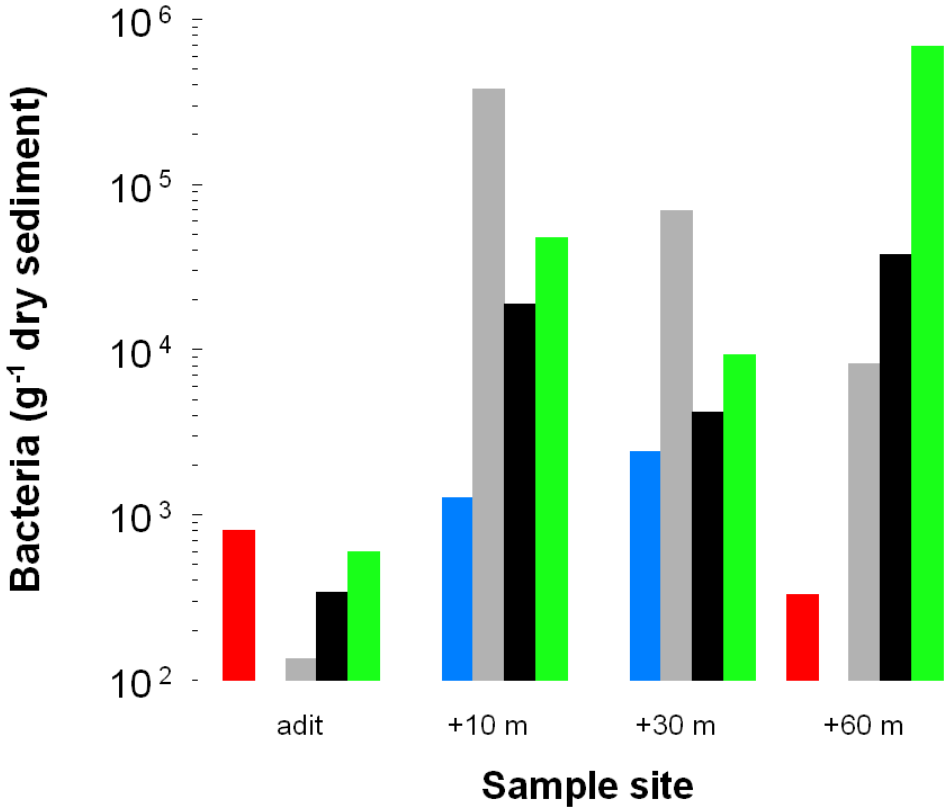

Fig. 3. Relative numbers of acidophilic bacteria isolated on solid media from sediment samples taken from the abandoned Cantareras copper mine, at and downstream from the mine adit entrance. Key: red bars, iron-oxidizing aerobes; blue bars, aerobic heterotrophs; grey bars, aSRB (isolated on glycerol/zinc plates); black bars, aSRB (isolated on glycerol copper plates, pH ~2.8); green bars, non-sulfidogenic anaerobes. No colonies were obtained on more acidic (pH ~2.3) glycerol/copper plates, or on circum-neutral pH solid media. In cases where bars are absent, numbers of bacteria isolated were less than 10^2 g^{-1}.

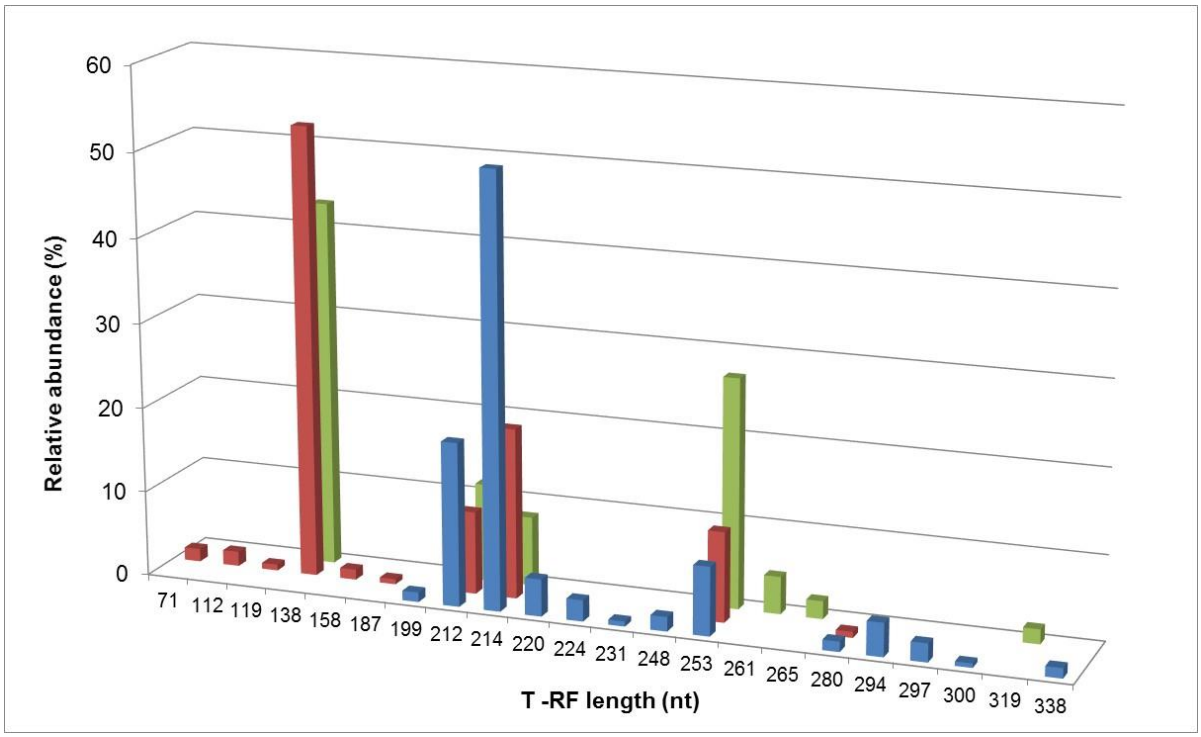

Fig. 4. Terminal restriction enzyme fragment length polymorphism (T-RFLP) profiles of bacterial 16S rRNA genes (digested with HaeIII) of planktonic bacteria in an acidic sulfidogenic bioreactor, operated at pH 4.5 (blue bars), pH 3.0 (red bars) and pH 2.8 (green bars).The main T-RFs corresponded to; 138 nt, *Peptococcaceae* strain CEB3; 212 nt, *Alicyclobacillaceae* strain IR2; 214 nt, *D. acididurans*; 253 nt, *At. ferrooxidans*.